Cytological diagnosis of endometritis in the mare: investigations of sampling techniques and relation to bacteriological results

Walter, Jasmin ; Neuberg, Klaus-Peter ; Failing, Klaus ; Wehrend, Axel

Abstract: Aim of this study was to compare uterine smears made using the Knudsen catheter, the cytology brush and a uterine culture swab with regard to diagnostic usefulness and the occurrence of neutrophils. Additionally correlation between culture results and the occurrence of neutrophils in uterine smears was investigated. Samples were collected from 340 mares, 81.5% of which were in estrus. Smears made using the cytology brush yielded more endometrial cells per high-power field than those made using the other two instruments ($p<0.0001$), and a larger proportion had PMNs compared with smears made using the uterine swab ($p<0.0001$). For smears made with the cytology brush, cultures of -hemolytic streptococci were more often ($p=0.002$) accompanied by PMNs than cultures of bacteria other than -hemolytic streptococci, and there was a positive correlation ($r(s)=0.2$ $p=0.01$) between the number of PMNs in smears and the number of colonies of -hemolytic streptococci. The cytology brush was superior to the other methods because it generated a larger proportion of diagnostic useful smears and the occurrence of PMNs in smears was significantly correlated with the occurrence of cultures of -hemolytic streptococci.

DOI: https://doi.org/10.1016/j.anireprosci.2012.05.012

Cytological diagnosis of endometritis in the mare: Investigations of sampling techniques and relation to bacteriological results

J Walter[a], KP Neuberg[b], K Failing[c], A Wehrend[b]

[a]Corresponding Author: Birkhof Stud, D 73072 Donzdorf, Germany

Present address: Clinic of Reproductive Medicine, Vetsuisse-Faculty University Zurich, Winterthurerstrasse 260, CH 8057 Zurich, Switzerland, phone: ++41-44-6358288, fax: ++41-44-6358904, email: jwalter@vetclinics.uzh.ch

[b]Clinic for Obstetrics, Gynaecology and Andrology of Large and Small Animals with Veterinary Ambulance, Justus-Liebig-University, Frankfurterstrassse 106, D 35392 Giessen, Germany, phone +49-641-99-38700, email: Axel.Wehrend@vetmed.uni-giessen.de, kp.neuberg@gmx.de

[c]Unit for Biomathematics and Data Processing, Justus-Liebig-University, Frankfurterstrassse 95, D 35392 Giessen, Germany, phone +49-641-99-38800, email: Klaus.Failing@vetmed.uni-giessen.de

Abstract

Aim of this study was to compare uterine smears made using the Knudsen catheter, the cytology brush and a uterine culture swab with regard to diagnostic usefulness and the occurrence of neutrophils. Additionally correlation between culture results and the occurrence of neutrophils in uterine smears was investigated. Samples were collected from 340 mares, 81.5% of which were in estrus. Smears made using the cytology brush yielded more endometrial cells per high-power field than those made using the other two instruments ($p < 0.0001$), and a larger proportion had PMNs compared with smears made using the uterine swab ($p < 0.0001$). For smears made with the cytology brush, cultures of β-hemolytic streptococci were more often ($p = 0.002$) accompanied by PMNs than cultures of bacteria other than β-hemolytic streptococci, and there was a positive correlation ($r_s = 0.2$ $p = 0.01$) between the number of PMNs in smears and the number of colonies of β-hemolytic streptococci. The cytology brush was superior to the other methods because it generated a larger proportion of diagnostic useful smears and the occurrence of PMNs in smears was significantly correlated with the occurrence of cultures of β-hemolytic streptococci.

Keywords: endometrial cytology, swabbing, bacteriology, endometrial smear, streptococci, E. coli

Abbreviations: PMN, polymorphonuclear cells

1. Introduction

Bacterial endometritis is considered the most important reproductive disease in the mare and is associated with considerable economic loss (Troedsson, 1997; Causey, 2006). However, in non-venereal bacterial endometritis, risk factors such as compromised uterine defense mechanisms or poor perineal conformation, rather than the bacterial organisms, constitute the primary problem (Causey, 2006). Persistent uterine infections caused by β-hemolytic streptococci and *E. coli* from the vagina are a common sequel to compromised uterine defense mechanisms (Causey, 2006; Klein et al., 2009). Cytological and bacteriological examinations of uterine swabs are the mainstay of diagnosis of acute endometritis in the mare (Walter and Wehrend, 2007), and several studies have shown a strong correlation between bacteriological and cytological findings (Knudsen, 1964; Lauer, 1977; Knudsen, 1982; Brook, 1985; La Cour and Sprinkle, 1985). With bacteriological examination alone, there is a risk of incorrect interpretation of the findings because of false-negative and false-positive culture results (Waelchli et al., 1988; Moller Nielsen, 2005; LeBlanc and Causey, 2009). Furthermore, a positive culture may not be associated with endometrial inflammation and reduced fertility (Moller Nielsen, 2005; LeBlanc et al., 2007). Cytological examination of a uterine sample is an extremely useful diagnostic tool, and several authors have placed its sensitivity above that of bacteriological examination (Wingfield Digby, 1978; Mattos et al., 1984; Riddle et al., 2007). There are various ways to classify cytological results; some authors record the number of PMNs as a percentage of all cells seen in a smear (Couto and Hughes, 1985; Ricketts and Mackintosh, 1987) while others record actual numbers of cells seen per microscopic field examined (Knudsen, 1964; Purswell et al., 1989). Likewise, there are several methods of procurement of uterine samples, including non-guarded and guarded uterine culture swabs, the cap attached to the end of a guarded culture instrument, uterine lavage samples and cytology brushes (Wingfield Digby, 1978; Couto and Hughes, 1984; La Cour and Sprinkle, 1985; Ball et al., 1988). In an early comparative study of various sampling methods, the quality of smears with respect to cell numbers and cell morphology was better in specimens collected with cytology brushes than in specimens obtained with cotton swabs (1997). The primary aim of the present investigation was to compare the

Knudsen catheter, a uterine culture swab and a commercial cytology brush for collection of uterine cytology samples with respect to diagnostic usefulness and numbers of PMNs in the sample. Another point was to examine the correlation between culture results and the occurrence of PMNs in uterine smears.

2. Materials and Methods

2.1. Animals

A total of 340 warmblood mares from a stud farm in Donzdorf, Germany were used for the study, which was undertaken in 2008 during the breeding season from early March to early August. The mares were bred artificially and some were sampled more than once during subsequent estrous cycles. Indications for sampling were clinical noticeable problems as discharge or reddening of vestibular or vaginal mucosa as well as being barren for one or more cycles. This resulted in a selection of mares potentially susceptible for uterine infections. Each sampling was preceded by collection of anamnesis and a thorough reproductive examination that included teasing, inspection of the perineum and trans rectal palpation and ultrasonography. As mares were presented for artificial insemination 81.5% were in estrus at the time of sampling, the remaining 18.5% were in diestrus.

2.2. Comparisons

2.2.1. Comparison of three methods for collecting cytological samples

Samples were collected using the Knudsen catheter (Laborgeräte Reinke, Giessen, Germany), a uterine culture swab (Minitube, Tiefenbach, Germany) and a cytology brush (Minitube, Tiefenbach, Germany). In 318 mares, double samples were collected using two methods: Knudsen catheter and cytology brush (n = 110), Knudsen catheter and uterine swab (n = 90) as well as cytology brush and uterine swab (n = 118).

2.2.2. Comparison of culture and cytological results

88 A total of 279 samples were collected for bacteriological culture. Samples were taken using the Knudsen
89 catheter or the uterine swab. Of the cytology samples used for comparison with the bacteriological results,
90 120, 160 and 119 were collected with the Knudsen catheter, cytology brush and uterine swab, respectively.
91
92 2.3. Collection of cytological and bacteriological samples
93 The mares were placed in an examination chute and a tail wrap was applied and the tail was hold to the side.
94 The perineum was first cleaned with dry paper towels and then with a disinfecting solution containing
95 propanol and benzalkonium chloride (Cutasept®, Bode Chemie GmbH, Hamburg), followed by thorough
96 drying with paper towels. The Knudsen catheter (Figure 1) was used to collect samples for cytological and
97 bacteriological examination. This is a guarded uterine instrument that can be autoclaved and used repeatedly;
98 it consists of a metal tube (87 cm length) and an inner spiral metal rod. The tip of the rod is designed such
99 that withdrawal of the rod into the tube seals the inside of the tube. Near the tip of the spiral rod is a hole
00 through which a small cotton swab is threaded. The tube includes a thickened area (olive), which marks the
01 area to rest the catheter in the outer orifice of the cervix. The catheter was introduced into the uterus
02 manually via the vagina and cervix with the spiral rod withdrawn maximally into the metal tube, until the
03 olive lies in the caudal cervix. The tip of the rod with the cotton swab was then advanced into the uterine
04 lumen and left in contact with the endometrium for 30 seconds. The rod was then withdrawn into the outer
05 tube and the instrument removed from the mare. The cotton swab was transferred to a small tube containing
06 sterile saline solution using sterile forceps. The material that was attached to the spiral rod was carefully
07 removed and placed on a glass slide. The uterine swab was an individually wrapped double-guarded culture
08 instrument designed for single use (Figure 1). It consisted of an outer protective plastic tube and a smaller
09 inner plastic tube that held a plastic rod to which the swab was attached. The tip of the outer tube was closed
10 but could be opened by applying pressure to the inner tube. The instrument was manually introduced into the
11 uterus, the inner tube was then pushed into the uterus and the swab was advanced and held in contact with
12 the endometrium for 30 seconds. The swab was then withdrawn into the inner and outer protective tube and

the instrument removed from the mare. The swab was then completely withdrawn and removed from the instrument and rolled onto a sterile glass slide (76 × 26 mm, iDL, Nidderau). The swab was then transferred to AMIES® transport medium (MEUS S.r.l., Piove di Sacco, Italy) and submitted for culture. The cytology brush consisted of a brush, attached to a plastic rod within a protective tube (Figure 1). When the cytology brush was used in combination with the uterine swab, the brush was introduced into the uterus via the outer tube of the swabbing instrument, which remained in the uterus. The brush was advanced into the uterus and the rod that held the brush was rotated carefully three times, so that enough cells were collected. The brush was then withdrawn into the protective tube and the instrument removed from the mare. To make a smear, the brush was rolled onto a glass slide using slight pressure. Cytological smears were air dried for a few minutes before they were fixed with methyl alcohol and stained using eosin and thiazine according to the manufacturer's instructions (Hemacolor® stain; Merck, Darmstadt). Stained smears were air dried and mounted (Walter and Wehrend, 2009; Walter et al., 2011).

2.4. Evaluation

2.4.1. Cytological examination

Smears were first scanned at 200x magnification to assess cellular distribution and morphology using a light microscope (Ortholux ll; Leica, Wetzlar). Deformation (of cells) was defined as clumps of cells, accumulation of unidentified cells, or single severely deformed cells that could not be allocated to an identifiable cell type. The diagnostic value of cell morphology was categorized as follows: grade 1, very good diagnostic value (< 10% of cells deformed); grade 2, good diagnostic value (10 to 20% of cells deformed); grade 3, poor diagnostic value (20 to 50% of cells deformed) and grade 4, no diagnostic value (> 50% of cells deformed). Seven high-power fields (400x) were then assessed in each smear and the epithelial cells and PMNs were counted. Smears with fewer than 35 cells per seven fields were not included in the analysis. The neutrophil index according to Ricketts and Mackintosh (Ricketts and Mackintosh, 1987) was

then calculated based on the numbers of the two cell types. Smears with over 0.5% PMNs were classified as positive. The number of red blood cells per high-power field was assessed semi quantitatively and classified as none, few, numerous and massive numbers.

2.4.2. Bacteriological examination

After collection, the samples were stored at 4°C until transportation to the laboratory[1]. Processing took place within 24 hours according to standard laboratory techniques (Klein et al., 2009). Swabs were plated onto blood agar and Gassner agar plates (Water-blue-metachrome-yellow lactose agar according to Gassner, 101282, Merck, Darmstadt) and also incubated in an enrichment medium (enrichment broth with 10% serum, Merck, Darmstadt) for 24 hours before inoculation onto blood agar and Gassner agar plates. Culture media were incubated aerobically at 37°C, examined at 24 hours and then re-incubated for another 24 hours. Identification of aerobic pathogens was based on appearance of colonies and biochemical characteristics. The number of colonies on primary plates was assessed semi quantitatively and scored as absent (no colonies), small numbers (1 to 50 colonies), moderate numbers (51 to 200 colonies) and large numbers (> 200 colonies) (Klein et al., 2009). Classification of non-venereal disease organisms into pathogenic and non-pathogenic bacteria is shown in Table 2 (Pohl et al., 1977; Ricketts, 1981; Ricketts and Mackintosh, 1987; Hinrichs et al., 1988; Klein et al., 2009).

For comparison of bacteriological and cytological results, the cytological findings were analyzed with respect to individual bacteriological organisms, negative cultures and cultures with pathogenic and non-pathogenic bacteria. The effects of individual bacterial organisms in pure or mixed cultures with pathogenic or non-pathogenic bacteria on cytological findings were analyzed. Numbers of colonies were correlated with cytology grades (Ricketts and Mackintosh, 1987).

2.5. Statistical analysis

The statistical software program packages BMDP/Dynamic, release 7.0 (Dixon, 1993) and BiAS (Ackermann, 1998) were used. Differences among cytological sampling techniques and between bacteriological and cytological findings were analyzed using contingency tables, created with the program BMDP4F, and tested for independency by the generalised Fisher exact test (Fisher-Freeman-Halton test) or the chi-square test. By the McNemar test symmetry was tested when significant differences ($p \leq 0.05$) were found. Differences between cytological techniques with respect to the diagnostic value of smears, cell deformation and occurrence of red blood cells were analyzed using a chi-square test and subsequently the configural frequency analysis (program BiAS) to detect patterns in the data that occur significantly more or significantly less often than expected by chance. Differences among cytological sampling techniques with respect to numbers of epithelial cells and PMNs per high-power field were analyzed using the Wilcoxon-Mann-Whitney-test (BMDP3D). The effect of recovery of individual organisms on cytological results was analyzed using the two-sided Fisher's exact test. Correlations between cytological techniques with respect to numbers of epithelial cells per high-power field, and between numbers of colonies and neutrophil index were analyzed using the Spearman rank correlation coefficient (BMDP3D).

3. Results

3.1. Comparison of cytological sampling techniques

There was a significant association ($p < 0.0001$) between the cytological technique and the proportion of diagnostic valuable samples (see Table 1); the Knudsen catheter yielded significantly more samples that were not analyzable because of insufficient cell numbers compared with the other two techniques, and the uterine swab yielded significantly more samples with deformed cells. The cytology brush achieved the greatest proportion (98.6%) of analyzable samples. More than 90% of samples collected with the uterine swab had cellular deformation compared with 25% of samples collected using the Knudsen catheter and cytology brush (Table 1). The statistical hypothesis that cell deformation is independent of the sampling

technique was rejected ($p < 0.0001$).

There were only 25 of 215 cytology brush specimens without red blood cells, which was significantly less than expected by equality between the methods used (Table 1). Therefore, the statistical hypothesis that the occurrence of red blood cells in cytological smears is independent of the sampling technique was rejected with a chi-square value of 163.5 ($p < 0.0001$).

3.2. Pair-wise comparison of cytological sampling techniques

3.2.1. Knudsen catheter versus cytology brush

A total of 66 paired smears were available for this comparison. The cytology brush yielded significantly more epithelial cells per high-power field than the Knudsen catheter (47.9 ± 14.4, median 48.3 versus 26.8 ± 15.8, median 22.6; $p < 0,0001$). On the other hand, the Knudsen catheter yielded significantly more PMNs per high-power field than the cytology brush (5.3 ± 14.7, median 0 versus 2.5 ± 10.6, median 0; $p < 0.02$). Sixteen (24%) paired smears yielded positive results (> 0.5% of all cells were PMNs), 29 pairs (44%) yielded negative results, 14 pairs (21%) yielded positive and negative results for the Knudsen catheter and cytology brush, respectively, and the remaining 7 pairs (11%) yielded negative and positive results for the Knudsen catheter and cytology brush, respectively. The two techniques did not differ ($p = 0.19$) with respect to the detection of PMNs > 0.5%.

3.2.2. Knudsen catheter versus uterine swab

A total of 43 paired smears were available for this comparison. The Knudsen catheter yielded a mean of 21.2 ± 15.1 (median 15.9) epithelial cells per high-power field and the uterine swab a mean of 19.2 ± 7.8 (median 17.7; $p = 0.89$) cells. The Knudsen catheter yielded significantly more PMNs per high-power field than the uterine swab (5.5 ± 14.8, median 0.1 versus 0.5 ± 3.2, median 0; $p < 0.0001$). Five paired smears (12%) yielded positive results (> 0.5% of all cells were PMNs), 22 pairs (51%) yielded negative results and the remaining 16 pairs yielded positive and negative results for the Knudsen catheter and uterine swab,

respectively. Significantly more cases of endometritis were detected using the Knudsen catheter than using the uterine swab ($p = 0.0001$).

3.2.3. Cytology brush versus uterine swab

A total of 89 paired smears were available for this comparison. The cytology brush yielded a mean of 45.8 ± 15.8 (median 45.6) epithelial cells per high-power field and the uterine swab 19.6 ± 9.9 (median 17.1; $p < 0.0001$) cells. The cytology brush yielded significantly more PMNs per high-power field than the uterine swab (4.4 ± 11.7, median 0.3 versus 0.6 ± 2.9, median 0; $p < 0.0001$). Eighteen paired smears (20%) yielded positive results (> 0.5% of all cells were PMNs), 44 pairs (50%) yielded negative results and the remaining 27 pairs (30%) yielded positive and negative results for the cytology brush and the uterine swab, respectively. Significantly more cases of endometritis were detected using the cytology brush than using the uterine swab ($p = 0.0001$).

3.3. Relationship between cytological and bacteriological results

The frequency with which the culture of a bacterial species was accompanied by a positive cytological result (> 0.5% of all cells were PMNs) was calculated for each of the three sampling techniques (Table 2). With the cytology brush, but not with the other two methods, there were significantly more smears with a positive cytological result when the smear was accompanied by a culture of β-hemolytic streptococci compared with cultures negative for these bacteria. Based on the results shown in 3.1. and 3.2., only the cytological specimens made using the cytology brush were used for further analysis. There was a trend ($p = 0.09$) for an association between culture result and occurrence of positive cytological result: 20% of cultures that yielded no growth (n = 30), 43% of cultures with non-pathogenic bacteria (n = 35) and 40% of cultures with pathogenic bacteria (n=95) were accompanied by a positive cytological result. Seven of 10 (70%) pure cultures of β-hemolytic streptococci were accompanied by a positive cytological result compared with 47% of cultures of β-hemolytic streptococci mixed with pathogenic (n = 34) and 37% of cultures of β-hemolytic

streptococci mixed with non-pathogenic bacteria (n = 8) (p = 0.34). Six of 16 (37%) pure cultures of *E. coli* were accompanied by a positive cytological result compared with 42% of cultures of *E. coli* mixed with pathogenic (n = 31) and 13% of cultures of *E. coli* mixed with non-pathogenic bacteria (n = 15) (p= 0.15). There was a significant association between the number of colonies of β-haemolytic streptococci, but not in cultures of other organisms, and the number of PMNs in smears ($r_s = 0.20$; $p = 0.01$).

4. Discussion

4.1. Comparison of cytological sampling techniques

In view of the enormous diagnostic usefulness of uterine cytology in equine reproduction, it is critical that the techniques and instrumentation used consistently yield smears with a sufficient number of morphologically intact cells for diagnostic purposes. For this reason, three different sampling techniques were evaluated with respect to the diagnostic value of the smear and cell morphology. The Knudsen catheter technique yielded the greatest proportion of non-diagnostic smears because of a paucity of cells. This may have been due to the relatively smooth surface of the metal spiral, which may have prevented adequate uptake of endometrial secretion, especially in a dry uterus. When adequate amounts of secretion were collected, the Knudsen catheter technique yielded smears of good quality and the largest percentage (76.3%) of specimens without cell deformation. Of the smears made from uterine swabs, 21.9% were not analyzable, mainly because of large numbers of deformed cells (n = 37, 18.4%), but also because of insufficient cell numbers (n = 7, 3.5%). Of the analyzable smears made from uterine swabs 91.1% had > 10% deformed cells; deformation was mild in 40.1% and pronounced in 51.0%. It is known that cotton swabs lead to cell deformation (Britton, 1982; Couto and Hughes, 1984; Brook, 1985; Bourke et al., 1997; Dascanio et al., 1997). Damage is thought to occur mainly during cell uptake in the uterus and when the swab is rolled onto the slide (Couto and Hughes, 1984; Dascanio et al., 1997). Too little pressure on the swab during the rolling

procedure may lead to clumping of cells, and excessive pressure may result in cell deformation and damaged cell nuclei (Britton, 1982). To increase the quantity and morphologic quality of cells in smears, several authors recommend wetting the cotton swab with sterile saline solution before collection (Merkt and Frhr. von Lepel, 1970; Pohl et al., 1977; Crickman and Pugh, 1986; Saltiel et al., 1987; Waelchli et al., 1988; Dascanio et al., 1997; Dascanio, 2003). Furthermore, it has been proposed that dry cotton has water-repellent properties and may therefore be limited for the collection of secretions (Merkt and Frhr. von Lepel, 1970). Older studies (Allen, 1979; Couto and Hughes, 1984; Brook, 1985; Dascanio, 2003) indicated that calcium-alginate swabs may yield better cytological specimens, but comparison of dry and wet swabs has not been done in the mare. All but three smears (insufficient cell numbers) made using the cytology brush were diagnostic, and this technique was superior with respect to diagnostic usefulness and cell morphology, confirming previous preliminary findings in mares and cows (Bourke et al., 1997; Kasimanickam et al., 1999).

The occurrence of red blood cells in cytological smears was considered an indication of the invasiveness of the technique (Bourke et al., 1997). Red blood cells in uterine smears are commonly seen and are usually attributable to trauma caused by the sampling procedure (Wingfield Digby, 1978; Dascanio et al., 1997). They have no specific diagnostic value, and therefore rather than counting them we assessed their numbers semiquantitatively. Only 11.6% of smears made using the cytology brush were free of red blood cells compared with 69.6% and 71.3% of smears made using the Knudsen catheter and uterine swab, respectively. Large numbers of red blood cells are commonly seen in smears from mares after foaling [14] and occasionally during estrus (Couto and Hughes, 1984), but can also indicate endometritis (Wingfield Digby, 1978). It is not surprising that the cytology brush is more invasive than the other two techniques, considering its mode of action; our findings confirm those of other studies (Wingfield Digby, 1978; Bourke et al., 1997). It should be noted, that the disruption of the epithelial lining, which is indicated by the presence of erythrocytes in the smears, might also result in a higher number of PMN in the smear.

87 4.2 Pair-wise comparison of cytological sampling techniques

88 The cytology brush was superior to the other two sampling techniques with respect to proportion of
89 diagnostic samples, preservation of cell morphology and the detection of PMNs. Almost all smears were
90 diagnostic, pronounced cell deformations were rare, and PMNs were detected significantly more frequently
91 than when the uterine swab was used. Approximately one third of smears obtained using the Knudsen
92 catheter were non-diagnostic because of limited cell numbers, which limits it's use for uterine cytology in the
93 mare.

94 Smears made using the cytology brush yielded significantly more epithelial cells per high-power field
95 than smears made using the Knudsen catheter and uterine swab; smears made using the latter two techniques
96 did not differ with respect to numbers of epithelial cells. In a previous study involving fewer mares, the
97 cytology brush was also superior to a cotton swab (Bourke et al., 1997). Because of the greater uptake of
98 epithelial cells by the cytology brush, it was reasonable to expect an increase in the number of PMNs as well.
99 However, this was only true when compared with the uterine swab; smears made using the Knudsen catheter
00 yielded more PMNs per high-power field than the cytology brush. Compared with the brush, the Knudsen
01 instrument has a larger surface area for uptake of secretions, which may explain the higher yield of PMNs
02 (Figure 1). This leads to the conclusion, that the Knudsen catheter is a good instrument to diagnose PMNs

03 In a study comparing the usefulness of the cytology brush and uterine swab for the detection of PMNs,
04 the latter method was found to have a higher sensitivity (Bourke et al., 1997). Our results showed that
05 compared to the other two techniques, the uterine swab had considerable disadvantages with respect to the
06 detection of PMNs and cell morphology.

07

08 4.3. Relationship between cytological and bacteriological results

09 One goal of this study was to investigate the relationship between the culture of pathogenic bacteria from the
10 uterus and the presence of PMNs in uterine smears. The recovery of bacteria from the uterus is not
11 necessarily indicative of endometritis (Kenney, 1978; Hughes et al., 1980), and therefore the results of

concurrent cytological examinations are used to interpret the bacteriological findings (Tillmann and Meinecke, 1980). There have been numerous studies that documented correlations of varying degrees between the two diagnostic tools (Knudsen, 1964; Pohl et al., 1977; Tillmann and Meinecke, 1980; Knudsen, 1982; Wingfield Digby and Ricketts, 1982; Couto and Hughes, 1984; Mattos et al., 1984; Brook, 1985; La Cour and Sprinkle, 1985; Ball et al., 1988; Roszel and Freeman, 1988; Brook, 1992; Langoni et al., 1997; Wehrend et al., 2004; Riddle et al., 2007). However, direct comparison between studies may be difficult because different cut-offs have been used to differentiate positive and negative cytological samples. Furthermore, some authors differentiated pure and mixed cultures (Wingfield Digby and Ricketts, 1982) while others did not, and non-guarded swabbing systems were used in some studies, which affected the results of both bacteriological and cytological examinations (Shin et al., 1979; Blanchard et al., 1981; Wingfield Digby and Ricketts, 1982; Aguilar et al., 2006; Klein et al., 2009). Due to this guarded instruments were used in this study.

The occurrence of PMNs was twice as common in smears accompanied by a positive culture compared with those accompanied by a negative culture, regardless of whether the organisms were pathogenic or not. This is a strong indication that a diagnosis of endometritis cannot be reliably made with culture results alone. Histological and microbiological examinations of endometrial biopsy specimens are considered gold standards (Moller Nielsen, 2005), but endometrial biopsy is a more involved procedure than routine culture and cytology. In a recent study on the comparison of different techniques for diagnosing subclinical endometritis, the sensitivities of the cytology brush and uterine swab were 0.17 and 0.00, respectively, and the specificities were 0.83 and 0.93, when biopsy results were used as the gold standard. In this experiment a higher limiting value (> 2% of all cells were PMNs) for positive cytologies was defined, most mares were sampled during diestrus, and only 11 of 55 mares had a positive culture; thus, a direct comparison with this findings is not possible (Overbeck et al., 2011).

In agreement with previous studies (Wingfield Digby and Ricketts, 1982; Katila et al., 1988; Watson, 2000; Causey, 2006; Wittenbrink et al., 2008), β-hemolytic streptococci were found to be the most important

non-venereal organisms cultured from the mare's uterus. Cytological smears made from the cytology brush that were accompanied by culture of β-hemolytic streptococci were significantly more often positive for PMNs than smears accompanied by other cultured organisms, regardless of whether the bacteria occurred in pure culture (70% had PMNs) or mixed with pathogenic (47%) or non-pathogenic bacteria (37%). Although the differences between these percentages were not significant, the findings suggest that pure cultures of β-hemolytic streptococci are more likely to cause an endometrial inflammatory response than mixed cultures. However, this is in contrast to one report in which 18% of pure and 22% of mixed cultures of β-hemolytic streptococci were accompanied by positive cytological findings (Wingfield Digby and Ricketts, 1982). Our results are the first to establish a significant positive correlation between the degree of uterine infection with β-hemolytic streptococci and the degree of neutrophilic response; however, further studies on this aspect of uterine infection in the mare are required.

In agreement with other studies (Riddle et al., 2007; Bindslev et al., 2008; Overbeck et al., 2011), positive cultures of *E. coli* were less often accompanied by positive cytological results than cultures of β-hemolytic streptococci. Our findings do not confirm the opinion that only pure cultures and large numbers of colonies of *E. coli* recovered from the mare's uterus are significant (Ricketts, 1981; Moller Nielsen, 2005). The number of colonies did not correlate with the number of PMNs in smears. The proportion of positive cultures that were accompanied by PMNs in smears was generally low, but more pure cultures than mixed cultures of *E. coli* with non-pathogenic bacteria had positive cytological results (37% versus 13%). The proportion of pure cultures of *E. coli* accompanied by PMNs in smears was even lower (slightly > 10%) in previous studies (Wingfield Digby and Ricketts, 1982; Waelchli et al., 1993). Thus, the interpretation of positive cultures of *E. coli* from the mare's uterus remains difficult.

5. Conclusions

Our study has shown that the cytology brush method is the technique of choice for cytological examination of the mare's uterus. Of the methods investigated, the cytology brush yielded the largest proportion of

diagnostic smears, and the numbers of PMNs recovered were statistically correlated with the numbers of colonies of β-hemolytic streptococci in concurrent uterine cultures. It seems to be necessary to initiate follow-up studies to investigate new guidelines for the interpretation of uterine smears prepared by cytology brushes.

Footnotes

[1]Chemical and Veterinary State Laboratory Stuttgart, Bacteriology, Schaflandstraße 3/3, 70736 Fellbach, Germany and Institute for Hygiene and Infectious Diseases of Animals, Justus Liebig University Gießen, Frankfurter Str. 89, 35392 Gießen, Germany

Acknowledgements

The authors thank Minitube (Tiefenbach, Germany) and Birkhof Stud (Donzdorf, Germany) for providing probands and equipment.

References

Ackermann, H., 1998. BiAS für Windows, Biometrische Analyse von Stichproben, Version 7.0. Epsilon-Verlag, Hochheim, Darmstadt.

Aguilar, J., Hanks, M., Shaw, D.J., Else, R., Watson, E., 2006. Importance of using guarded techniques for the preparation of endometrial cytology smears in mares. Theriogenology 66, 423-430.

Allen, W.E., 1979. Aspects of genital infection and swabbing techniques in the mare. Vet. Rec. 104, 228-231.

Ball, B.A., Shin, S.J., Patten, V.H., Lein, D.H., Woods, G.L., 1988. Use of a low-volume uterine flush for microbiologic and cytologic examination of the mare's endometrium. Theriogenology 29, 1269-1283.

Bindslev, M.M., Villumsen, M.H., Petersen, M.M., Bogh, I.B., Bojesen, A.M., 2008. Genetic diversity of *S. equi ssp. zooepidemicus* ane *E. coli* isolated from the reproductive tract of the mare. Reprod Dom Anim 43, 110-111.

Blanchard, T.L., Garcia, M.C., Hurtgen, J.P., Kenney, R.M., 1981. Comparison of two techniques for obtaining endometrial bacteriologic cultures in the mare. Theriogenology 16, 85-93.

Bourke, M., Mills, J.N., Barnes, A.L., 1997. Collection of endometrial cells in the mare. Aust. Vet. J. 75, 755-758.

Britton, B.A., 1982. Endometrial change in the annual reproductive cycle of the mare. J. Reprod. Fert. Suppl. 32, 175-180.

Brook, D., 1985. Cytological and bacteriological examination of the mare's endometrium. Equine Vet. Sci. 5, 16-22.

Brook, D., 1992. 28. Uterine Cytology, In: McKinnon, A.O., Voss, J.L. (Eds.), Equine Reproduction, Lippincott Williams & Wilkins, Philadelphia, pp. 246-254.

Causey, R.C., 2006. Making sense of equine uterine infections: the many faces of physical clearance. Veterinary Journal 172, 405-421.

Couto, M.A., Hughes, J.P., 1984. Technique and interpretation of cervical and endometrial cytology in the mare. Equine Vet. Sci. 4, 265-273.

Couto, M.A., Hughes, J.P., 1985. Intrauterine inoculation of a bacteria-free filtrate of streptococcus zooepidemicus in clinically normal and infected mares. Equine Vet. Sci. 5, 81-86.

Crickman, J.A., Pugh, D.G., 1986. Equine endometrial cytology: A review of techniques and interpretations. Vet. Med. July, 650-656.

Dascanio, J.J., 2003. Endometrial Cytology, In: Robinson, N.E. (Ed.), Current therapy in equine medicine, Saunders, Philadelphia, pp. 226-228.

Dascanio, J.J., Ley, W.B., Bowen, J.M., 1997. How to perform and interpret uterine cytology, Proc Am Assoc Equine Pract, pp. 182-186.

10 Dixon, W.J., 1993. BMDP Statistical Software Manual, Volume 1 and 2. University of California Press,
11 Berkeley, Los Angeles, London.
12 Hinrichs, K., Cumings, M.R., Sertrich, P.L., Kenney, R.M., 1988. Clinical significance of aerobic bacterial
13 flora of the uterus, vagina, vestibule, and clitorial fossa of clinically normal mares. J Am Med Assoc 193, 72-
14 75.
15 Hughes, J.P., Stabenfeldt, G.H., Kennedy, P.C., 1980. The estrous cycle and selected functional and
16 pathologic ovarian abnormalities in the mare. Vet. Clin. North. Am. Large Anim. Pract. 2, 295-298.
17 Kasimanickam, R., Leslie, K., Duffield, T., Gartley, C., Johnson, W., 1999. A comparison of the cytobrush
18 and uterine lavage technique to evaluate endometrial cytology in postpartum dairy cows, Proc 32th Ann
19 Meet Soc for Theriogenology, Nashville, Tennessee, p. 6.
20 Katila, T., Koskinen, E., Oijala, M., Parviainen, P., 1988. Evaluation of the post-partum mare in relation to
21 foal heat breeding. II. Uterine swabbing and biopsies. Zentralbl Veterinarmed A 35, 331-339.
22 Kenney, R.M., 1978. Cyclic and pathologic changes of the mare endometrium as detected by biopsy, with a
23 note on early embryonic death. J. Am. Vet. Med. Assoc. 172, 241-262.
24 Klein, C., Ennen, S., Huchzermeyer, S., Weiss, R., Wehrend, A., 2009. Analysis of the barrier function of
25 vulvovaginal fold and cervix to ascending bacterial contamination of the mare's reproductive tract. Tierarztl
26 Prax 2, 113-117.
27 Knudsen, O., 1964. Endometrial cytology as a diagnostic aid in mares. Cornell Vet 54, 415-422.
28 Knudsen, O., 1982. A combined cytologic and bacteriologic endometrial examination in the mare, Proc. Am.
29 Assoc. Equine Pract., pp. 431-433.
30 La Cour, A., Sprinkle, T.A., 1985. Relationship of endometrial cytology and fertility in the broodmare.
31 Equine Pract. 7, 28-36.
32 Langoni, H., Alvarenga, M.A., Papa, F.O., Sakamoto, C., Baldini, S., Listoni, F.J., 1997. Aerobic,
33 microaerobic and anaerobic bacteria in equine endometritis. Pferdeheilk. 13, 548.

Lauer, J., 1977. Die klinische Zytodiagnostik zur Verifizierung symptomarmer Genitalinfektionen bei der Stute, Ambulatorische und Geburtshilfliche Veterinärklinik, Justus-Liebig-Universität, Gießen, p. 96.

LeBlanc, M., Magsig, J., Stromberg, A.J., 2007. Use of a low-volume uterine flush for diagnosing endometritis in chronically infertile mares. Theriogenology 68, 403-412.

LeBlanc, M.M., Causey, R.C., 2009. Clinical and subclinical endometritis in the mare: both threats to fertility. Reprod Domest Anim 44 Suppl 3, 10-22.

Mattos, R.C., Mattos, A.L.G., Günzel, A.-R., Klug, E., 1984. Bakteriologische und zytologische Untersuchungen von Uterusabstrichen beim Pferd. Prakt. Tierarzt 10, 809-814.

Merkt, H., Frhr. von Lepel, J., 1970. Die Entnahme von Proben für die bakteriologische Untersuchung des Genitalsekretes der Stute, ihre Beurteilung sowie Behandlungshinweise. Dtsch Tierärztl Wschr 77, 489-528.

Moller Nielsen, J., 2005. Endometritis in the mare: a diagonstic study comparing cultures from swab and biopsy. Theriogenology 64, 510-518.

Overbeck, W., Witte, T.S., Heuwieser, W., 2011. Comparison of three diagnostic methods to identify subclinical endometritis in mares. Theriogenology 75, 1311-1318.

Pohl, R., Wagenseil, F., Hünermund, G., Romer, H., 1977. Die zytologische Untersuchung von Cervix-Tupferproben als diagnostisches Hilfsmittel bei der gynäkologischen Untersuchung und der zuchthygienischen Beurteilung von Stuten. Tierärztl. Umsch. 12, 634-640.

Purswell, B.J., Ley, W.B., Sriranganathan, N., Bowen, J.M., 1989. Aerobic and anaerobic bacterial flora in the postpartum mare. Equine Vet. Sci. 9, 141-144.

Ricketts, S.W., 1981. Bacteriological examinations of the mare's cervix: Techniques and interpretation of results. Vet. Rec. 108, 46-51.

Ricketts, S.W., Mackintosh, M.E., 1987. Role of anaerobic bacteria in equine endometritis. J Reprod Fert Suppl 35, 343-351.

Riddle, W.T., LeBlanc, M., Stromberg, A.J., 2007. Relationship between uterine culture, cytology and pregnancy rates in a thoroughbred practice. Theriogenology 68, 395-402.

59 Roszel, J.-F., Freeman, K.-P., 1988. Equine endometrial cytology. Vet Clin North Am Equine Pract 4, 247-
60 262.
61 Saltiel, A., Gutierrez, A., de Buen-Llado, N., Sosa, C., 1987. Cervico-endometrial cytology and
62 physiological aspects of the post-partum mare. J Reprod Fert Suppl 35, 305-309.
63 Shin, S.J., Lein, D.H., Aronson, A.L., Nusbaum, S.R., 1979. The bacteriological culture of equine uterine
64 contents: In-vitro sensitivity of organisms isolated and interpretation. J. Reprod. Fert. Suppl. 27, 307-315.
65 Tillmann, H., Meinecke, B., 1980. Die zytodiagnostische Interpretation der lokalen Abwehrreaktionen bei
66 Genitalinfektionen der Stute. Tierärztl. Praxis 8, 211-222.
67 Troedsson, M.H.T., 1997. Diseases of the uterus, In: Robinson, N.E. (Ed.), Current therapy in equine
68 medicine, Saunders, Philadelphia, pp. 517-524.
69 Waelchli, R.O., Corboz, L., Winder, N.C., 1988. Comparison of histological, cytological and bacteriological
70 findings in the endometrium of the mare. J. Vet. Med. A. 35, 442-449.
71 Waelchli, R.O., Känzig, M., Gygax, A., Corboz, L., Rüsch, P., 1993. The relationship between cycle stage
72 and results of uterine culture in the mare. J. Vet. Med. A. 40, 569-575.
73 Walter, J., Klein, C., Wehrend, A., 2011. Comparison of eosin-thiazin and Papanicolaou-Shorr staining for
74 endometrial cytologies of broodmares. Technical Note. Tierarztl Prax Ausg G Grosstiere Nutztiere 39, 358-
75 362.
76 Walter, J., Wehrend, A., 2007. Exfoliative endometrial cytology as a diagnostic aid in the gynaecological
77 examination of broodmares. Pferdeheilk. 23, 481-488.
78 Walter, J., Wehrend, A., 2009. Exfoliative Endometriumszytologie bei der Zuchtsute - Probenentnahme und
79 Befundinterpretation. Tierarztl Prax Ausg G Grosstiere Nutztiere 37, 409.
80 Watson, E.D., 2000. Post-breeding endometritis in the mare. Animal reproduction science 60-61, 221-232.
81 Wehrend, A., Huchzermeyer, S., Reinemund, B., 2004. Investigations on the suitability of intracervical and
82 intrauterine sampling for bacteriological investigations and cytological assessment in mares. Wiener
83 Tierärztl. Monatsschr. 91, 72.

Wingfield Digby, N.J., 1978. The technique and clinical application of endometrial cytology in mares. Equine Vet. J. 10, 167-170.

Wingfield Digby, N.J., Ricketts, S.W., 1982. Results of concurrent bacteriological and cytological examinations of the endometrium of mares in routine stud farm practice 1978-1981. J. Reprod. Fert. Suppl. 32, 181-185.

Wittenbrink, M.M., Hoelzle, K., Hoelzle, L.E., 2008. What's new in bacteriology of the mare's genital tract. Pferdeheilk. 24, 53-55.

Figure 1 Instruments for collection of uterine samples for bacteriological and cytological examination. Double-guarded uterine culture swab (top), which is also used for passing the Cytology brush into the uterus (middle), and guarded Knudsen catheter (bottom)

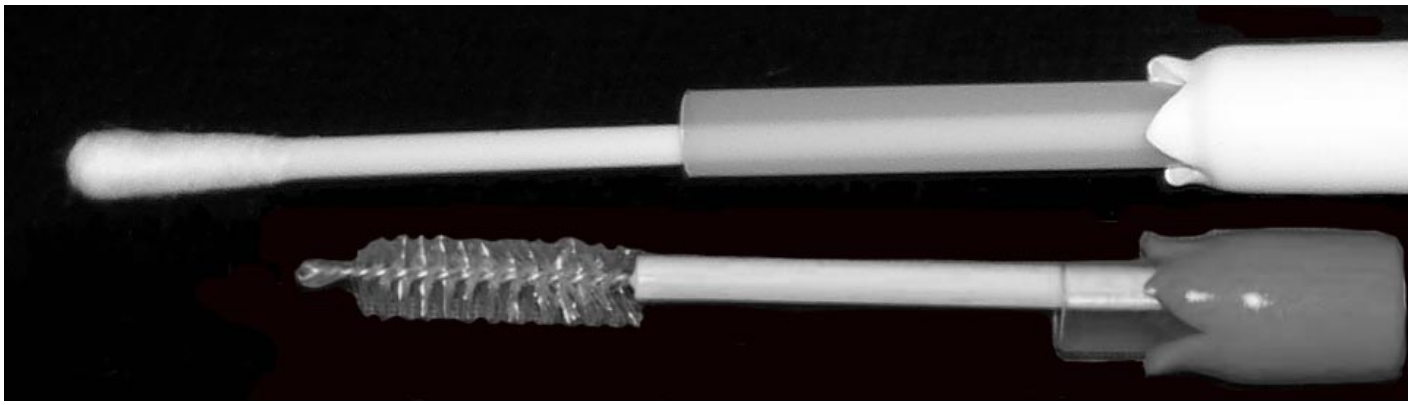

Table 1 Results of the configural frequency analysis of the diagnostic usefulness of samples (n = 626), occurrence of cellular deformation and red blood cells in diagnostic samples (n = 503). Absolute and relative numbers of samples (round brackets), expected cell frequencies by assumption of independency (square brackets) and p-values are given

Table 2 Paired bacteriological and cytological results of the 3 sampling methods. Associations between organism and cytological result were analyzed using the two-sided Fisher's exact test respectively the chi-square test depending of the size of the minimum expected value (AP, non-pathogenic bacteria; FP, pathogenic bacteria)

Category	Knudsen-catheter	Cytology brush	Uterine culture swab	Total
Not analyzable (insufficient cell numbers)	74 (35.7%) [27.8] p < 0.0001	3 (1.4%) [29.3] p < 0.0001	7 (3.5%) [27.0] p < 0.0001	84
Not analyzable (> 50% of cells deformed)	2 (1.0%) [12.9] p < 0.0017	0 (0%) [13.6] p < 0.0001	37 (18.4%) [12.5] p < 0.0001	39
Analyzable	131 (63.3%) [166.3] p < 0.0001	215 (98.6%) [175.23] p < 0.0001	157 (78.1%) [161.5] p < 2.9	503
Number of samples	207	218	201	626
Grade 1 <10% of cells deformed	100 (76.3%) [71.1] p < 0.0001	159 (74.0%) [116.7] p < 0.0001	14 (8.9 %) [85.2] p < 0.0001	273
Grade 2 10% to 20% of cells deformed	23 (17.6%) [36.2] p = 0.025	53 (24.7%) [59.4] p = 1.8	63 (40.1%) [43.4] p = 0.0002	139
Grade 3 20% to 50% of cells deformed	8 (6.1%) [23.7] p = 0.0003	3 (1.3%) [38.9] p < 0.0001	80 (51.0%) [28.4] p < 0.0001	91
Number of samples	131	215	157	503
No red blood cells	65 (69.6%) [52.5] p = 0.12	25 (11.6%) [86.3] p < 0.0001	112 (71.3%) [63.0] p < 0.0001	202
A few red blood cells	34 (26.0%) [35.4] p = 8.9	68 (31.6%) [58.1] p = 0.54	34 (21.7%) [42.4] p = 0.8	136
Numerous red blood cells	17 (13.0%) [28.9] p = 0.04	83 (38.6%) [47.4] p < 0.0001	11 (7.0%) [34.6] p <0.0001	111
Massive numbers of red blood cells	15 (11.4%) [14.1] p = 9.1	39 (18.2%) [23.1] p < 0.0001	0 (0%) [16.9] p < 0.0001	54
Number of samples	131	215	157	503

Bacterial organism		Cytology result Knudsen catheter		Cytology result Cytology brush		Cytology result Uterine culture swab	
		+	-	+	-	+	-
β-hemolytic streptococci (FP)	positive	16 (57%)	12 (43%)	26 (50%)	26 (50%)	5 14%	31 (86%)
	negative	38 (41%)	54 (59%)	33 (31%)	75 (69%)	10 (12%)	73 (88%)
p-Value		0.14		0.02		0.78	
E. coli (FP) positive	positive	14 (42%)	19 (58%)	21 (34%)	31 (66%)	5 (11%)	39 (89%)
	negative	40 (46%)	47 (54%)	38 (39%)	60 (61%)	10 (13%)	65 (87%)
p-value		0.73		0.53		0.75	
Hemolytic *E. coli* (FP)	positive	0 (0%)	1 (100%)	1 (100%)	0 (0%)	1 (100%)	0 (0%)
	negative	54 (45%)	65 (55%)	58 (37%)	101 (63%)	14 (12%)	104 (88%)
p-value		1.00		0.37		0.13	
Klebsiella spp. (FP)	positive	4 (50%)	4 (50%)	3 (43%)	4 (57%)	0 (0%)	7 (100%)
	negative	50 (45%)	62 (55%)	56 (37%)	97 (63%)	15 (13%)	97 (87%)
p-value		1.00		0.71		0.60	
Pseudomonas spp. (FP)	positive	4 (50%)	4 (50%)	3 (23%)	10 (77%)	0 (0%)	2 (100%)
	negative	50 (45%)	62 (55%)	56 (38%)	61 (42%)	15 (13%)	102 (87%)
p-value		1.00		0.28		1.00	
Staphylococcus aureus (FP)	positive	1 (100%)	0 (0%)	1 (100%)	0 (0%)	0 (0%)	1 (100%)
	negative	53 (45%)	66 (55%)	58 (36%)	101 (64%)	15 (13%)	103 (87%)
p-value		0.45		0.37		1.00	
Aerobic bacilli (AP)	positive	3 (43%)	4 (57%)	1 (25%)	3 (75%)	2 (40%)	3 (60%)
	negative	51 (45%)	62 (55%)	58 (37%)	98 (63%)	13 (11%)	101 (89%)
p-value		1.00		1.00		0.12	
Coliforms (AP)	positive	3 (33%)	6 (67%)	3 (37%)	5 (63%)	1 (7%)	14 (93%)
	negative	51 (46%)	60 (54%)	56 (37%)	96 (63%)	14 (14%)	90 (86%)
p-value		0.51		0.97		0.46	
α-hemolytic streptococci (AP)	positive	9 (45%)	11 (55%)	8 (31%)	18 (69%)	2 (10%)	19 (90%)
	negative	45 (45%)	55 (55%)	51 (38%)	83 (62%)	13 (13%)	85 (87%)
p-value		1.00		0.48		1.00	
γ-hemolytic	positive	5	10	8	11	3	10

streptococci (AP)		(33%)	(67%)	(42%)	(58%)	(23%)	(77%)
	negative	49 (47%)	56 (53%)	51 (36%)	90 (64%)	12 (11%)	94 (89%)
p-value		0.33		0.61		0.21	
Enterococcus spp. (AP)	positive	0 (0%)	1 (100%)	2 (50%)	2 (50%)	0 (0%)	3 (100%)
	negative	54 (45%)	65 (55%)	57 (37%)	99 (63%)	15 (13%)	101 (87%)
p-value		1.00		0.63		1.00	
Staphylococcus epidermidis (AP)	positive	6 (50%)	6 (50%)	5 (29%)	12 (71%)	2 (11%)	16 (89%)
	negative	48 (44%)	60 (56%)	54 (38%)	89 (62%)	13 (13%)	88 (87%)
p-value		0.71		0.50		0.84	
Acinetobacter spp. (AP)	positive	3 (25%)	9 (75%)	10 (53%)	9 (47%)	2 (18%)	9 (82%)
	negative	51 (47%)	57 (53%)	49 (35%)	92 (65%)	13 (12%)	95 (88%)
p-value		0.14		0.13		0.56	
Citrobacter spp. (AP)	positive	0 (0%)	3 (100%)	1 (100%)	0 (0%)	0 (0%)	3 (100%)
	negative	54 (46%)	63 (54%)	58 (37%)	101 (63%)	15 (13%)	101 (87%)
p-value		0.25		0.37		1.00	
Corynebacterium spp. (AP)	positive	2 (40%)	3 (60%)	2 (18%)	9 (82%)	0 (0%)	2 (100%)
	negative	52 (45%)	63 (55%)	57 (38%)	92 (62%)	16 (14%)	102 (86%)
p-value							
Micrococcus spp. (AP)	positive	-	-	-	-	0 (0%)	1 (100%)
	negative	-	-	-	-	16 (13%)	103 (87%)
p-value		-		-		1.00	
Alcaligenes spp. (AP)	positive	0 (0%)	1 (100%)	-	-	-	-
	negative	55 (46%)	65 (54%)	-	-	-	-
p-value		1.00		-		-	
Yeast (FP)	positive	-	-	0 (0%)	1 (100%)	-	-
	negative	-	-	59 (37%)	100 (63%)	-	-
p-value		-		1.00		-	
β-hemolytic streptococci and *E. coli* (FP)	positive	23 (49%)	24 (51%)	34 (40%)	51 (60%)	6 (10%)	56 (90%)
	negative	31 (42%)	42 (58%)	25 (33%)	50 (67%)	9 (16%)	48 (84%)
p-value		0.49		0.38		0.32	